REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1. Name and designation of ICMR- IF	: Dr. Srilekha Sundaramurthy Senior Scientist
2. Address	: Medical Research (Sankara Nethralaya) 18, College Road, Chennai 600006
3. Frontline area of research in which Training/research was carried out	: a) Whole Genome Sequencing Analysis in patients with suspected Inherited Optic Neuropathies (ION) b) Whole exome sequencing analysis in patients with suspected Leber Hereditary Optic Neuropathy (LHON) / ION c) Differentiation of *OPA1* mutated CRISPR-Cas9 corrected iPSC (from patient derived fibroblast) to Retinal progenitor cells d) Characterization of mitochondrial variants causative for ION
4. Name & address of Professor and host institute	: Prof. Patrick-Yu-Wai-Man Professor of Ophthalmology and Consultant Ophthalmologist John van Geest Centre for Brain Repair MRC Mitochondrial Biology Unit Department of Clinical Neurosciences University of Cambridge Cambridge, CB2 0PY, United Kingdom

5. Duration of fellowship with exact date : For a period of 6 months (March 27th 2023 – September 26th 2023)

6. Highlights of work conducted

Whole Genome Sequencing (WGS) in ION / LHON cases

ION are the most common cause of vision loss in early childhood with an estimated prevalence of about 1:10,000. The two main clinical presentations are Dominant Optic Atrophy (DOA) and Leber Hereditary Optic Neuropathy (LHON). **Whole genome sequencing (Illumina HiSeq platform) was conducted on eighteen suspected ION and three LHON cases. I was provided with access to the raw data by the host institute to allow me to gain technical experience in data analysis and for the mutual benefit of both institutions.**

- The potential disease causative variations were identified in **seventeen ION** and **two LHON families (90%) of which 25% were novel variations.**
- **Two deep intronic variations** were also identified from two different families and these need to investigate further by functional studies.
- Differential diagnosis and incidental findings were identified in **four different families (retinal / optic atrophy gene involvement).**
- A high level of genetic heterogeneity in the families were seen and **different modes of inheritance (autosomal dominant / recessive / digenic) were also identified.**
- One novel genes previously not linked with dominant optic atrophy have been identified as potential candidate gene ***(CACNA1C)***. However, segregation, and functional studies are required to confirm pathogenicity.

Whole exome sequencing in LHON like cases:

- Potential disease causative variations were identified in four LHON suspected families. Three families had variations in *OPA1* (c.C2506T: p.R836W); *OPA1* c.C2212A: p.R738S; *MFN2* (c.1039-2A>G).
- Of the four families one had a digenic inheritance linked with two different genes, namely, *NR2F1* and *SUCLA2*.
- The molecular diagnosis of these families will be helpful for genetic counselling and potential future treatments.

CRISPR-Cas9 mediated gene correction in iPSC cell-and differentiate into retinal progenitor cell

Primary fibroblasts were established from a skin biopsy donated by a patient carrying a pathogenic heterozygous *OPA1* variant (c.1334G>A: p.R445H). Cell reprogramming was done by episomal vectors encoding reprogramming factors followed by clonal iPSC isolation. CRISPR-Cas9 gRNAs and ssDNA template were designed using Benchling CRISPR design software. Individual iPSC colonies were manually selected and subjected to Sanger sequencing and western blot analysis. Furthermore, to confirm that *OPA1* gene correction had restored mitochondrial function, quantification of mitochondrial DNA copy number by qPCR was performed. Further differentiation of CRISPR-Cas9 corrected iPSC (c.1334G>A: p.R445H) wildtype was differentiated into Retinal progenitor cells was also observed.

Drug Therapeutics:

Drugs such as co-enzyme Q (CoQ10), sodium valproate and niacin (vitamin B3) were tested at various dose levels in wild-type zebrafish embryos. The proportion of dead embryos were then checked after 96 hours of incubation. CoQ10 is an antioxidant frequently given to patients with mitochondrial diseases. Sodium valproate is a drug targeting endoplasmic reticulum (ER) stress that has been shown to increase the expression of p21, which is crucial for cell survival

under conditions of heightened ER stress. Vitamin B3, which is a precursor for the synthesis of NAD+, has been shown to improve muscle performance in patients with mitochondrial myopathy. Determining the safety and efficacy of these drugs in wild-type and mutant zebrafish could be pave the way for future drug treatment trials in humans for various ION families.

i) **Technique/expertise acquired**:

Fig: 1 Whole genome sequencing: Overview of bioinformatics pipeline for Single Nucleotide Polymorphisms (SNPs) and small Insertions and Deletions (small InDels)

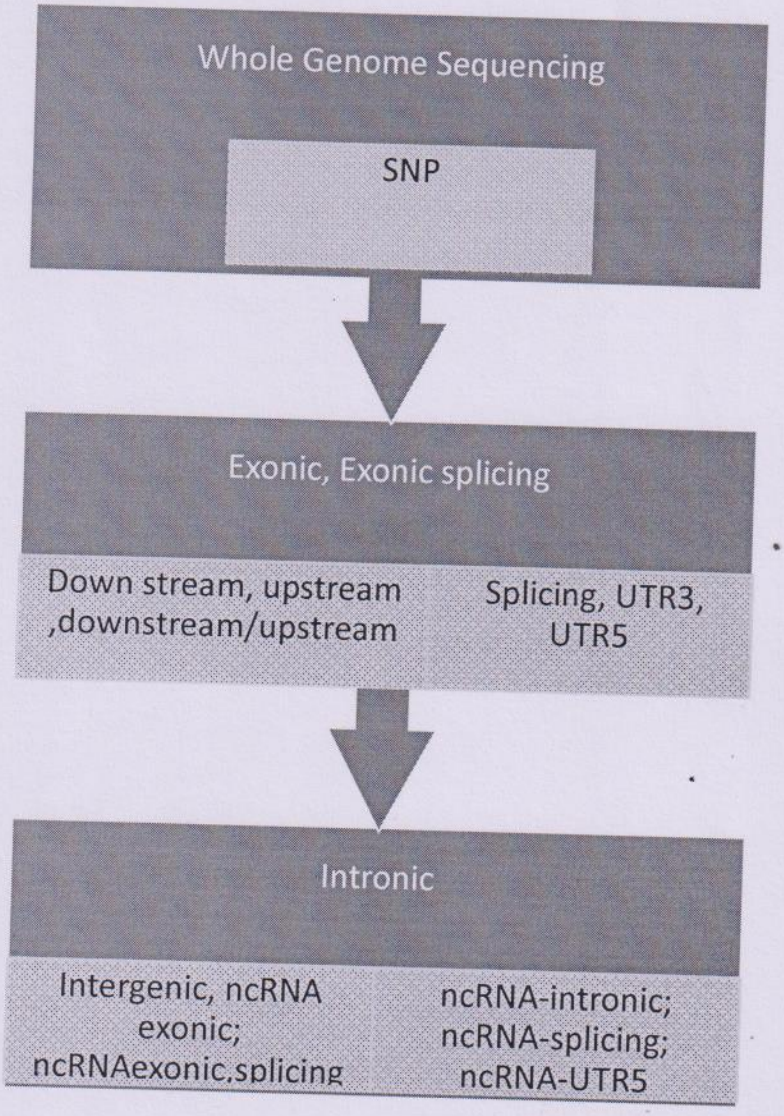
In brief, whole genome sequencing was done with Illumina Hiseq platform and data processing, alignment, variant calling and annotation was done according to the above-mentioned steps. GATK tool was used to call SNPs/InDels from BAM files and ANNOVAR to annotate variants.

Copy Number Variations (CNVs) are genomic variants leading to variation in copy number of relatively larger fragment (longer than 50bp) are detected. There are two types i.e gains and losses of copies. In this analysis, CNV detection was performed with the software Control-FREEC.

Structural variants (SVs) are genomic variants with relatively large size (>50bp), including deletions, duplications, insertions, inversions and translocations. SV detection and genotyping were performed with the software DELLY.

Further processing of the data was done by copying the data in sublime text and by transforming the data manually in CSV format. Further processing of data (SNP, Indel, SV, CNV) was done by following the below mentioned protocols.

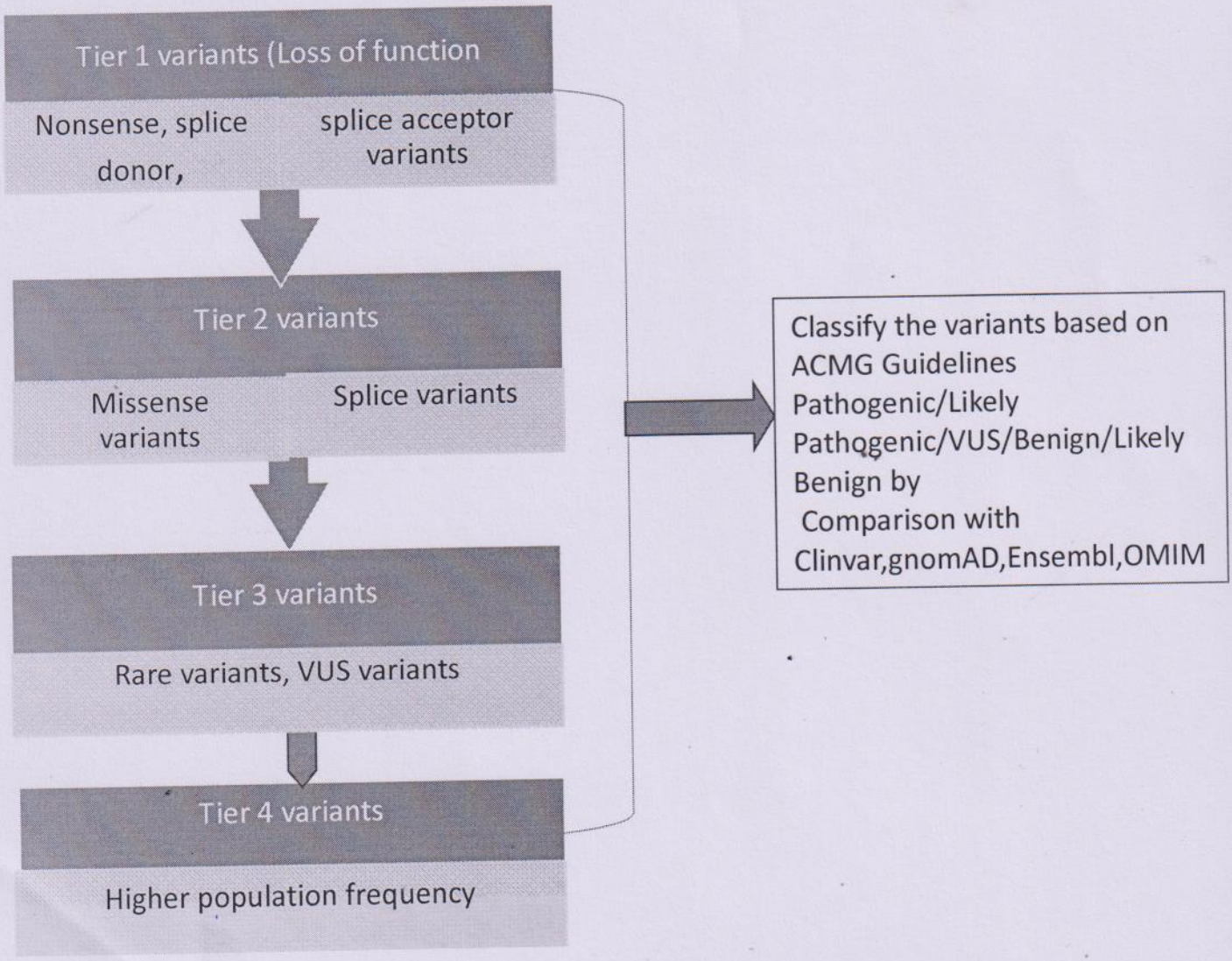
Fig: 2 Trimming of VCF files based on coding and noncoding regions



The data was processed based on the coding regions and non-coding regions and it was split in the same way for all the four different classes (SNP / InDels / CNV / SV) of variant files in CSV format.

Furthermore, the causative variants were prioritized from the known and unknown candidate genes following these guidelines. The Neuro-ophthalmology Panel (97 genes) Retinal Dystrophy Panel (351 genes) excluding the mitochondrial genes were analyzed. In cases where potential variants were not identified in known genes, variants were analyzed in genes based on their function and pathways (OMIM) related to mitochondrial maintenance.

Fig: 3 Overview of analyses



Characterization of mitochondrial variants:

Primary fibroblast culture was established from skin biopsy samples obtained from patients with mitochondrial diseases along with control fibroblast cultures were subjected to RNA extraction, cDNA analysis followed by RT-PCR was done to determine the expression levels of nuclear encoded gene compared between the wildtype and mutant cells.

Assessment of relative nuclear and mtDNA copy number by real time quantitative PCR (qPCR) inorder to know whether the nuclear gene mutations encoding a mitochondrial function relatively decreases the mtDNA copy number compared between the affected individuals with homozygous mutations and asymptomatic heterozygous carriers and with controls. The relative mitochondrial DNA content was determined using the equation: $\Delta Cq = nucDNA\ Cq - mtDNA\ Cq$

Assessment of mitochondrial membrane potential using tetramethylrhodamine ethyl ester (TMRE) in combination with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) inorder to measure the changes in the electrical potential across the inner membrane of the mitochondria in live cells.

Mitochondrial Network Analysis

Live fibroblasts were stained using MitoTracker CMXRos to visualize mitochondrial networks and then fixed and imaged using a confocal microscope. Wild type fibroblast is compared with the mutant and checked for mitochondrial network decrease in volume, length and the number of branches.

ii) **Research results, including any papers, prepared/submitted for publication:**

Table 1: Results of Whole Exome Analysis (LHON-like families)

S.No	Gene	Monoallelic / Biallelic	Reported / Novel
1	*OPA1*	Monoallelic	Reported
2	*OPA1*	Biallelic	Novel
3	*MFN2*	Monoallelic	Reported
4	*NR2F1* / *SUCLA2*	Monoallelic / Biallelic	Novel / Novel

Table 2: Results of Whole Genome Analysis (ION / LHON)

S.No	Gene	Monoallelic / Biallelic	Reported / Novel
1.	*KIF1B*	Monoallelic	Reported
2.	*NDUFS1* / *NDUFAF1*	Monoallelic / Monoallelic	Reported / Reported
3.	*MTPAP*	Biallelic	Novel
4.	*NR2F1*	Monoallelic	Novel
5.	*CACNA1C*	Monoallelic	Novel
6.	*ACO2*	Monoallelic	Reported
7.	*WFS1*	Biallelic	Reported
8.	*UCHL1*	Monoallelic	Novel
9.	*ACAD9*	Biallelic	Novel
10.	*WFS1*	Biallelic	Reported
11.	*AFG3L2*	Monoallelic	Novel

Fig: 4: Variants identified in LHON-like families (Whole exome & Whole genome analysis)

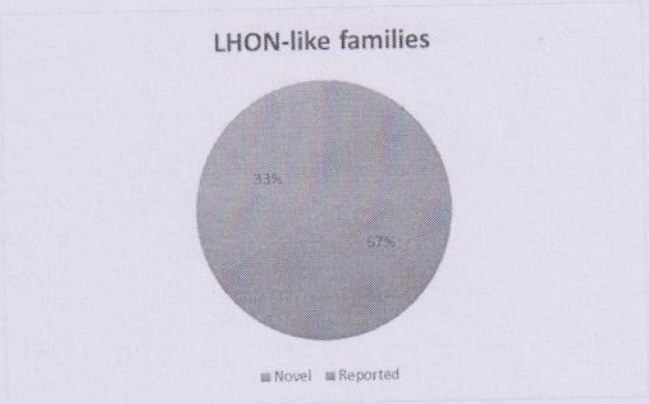
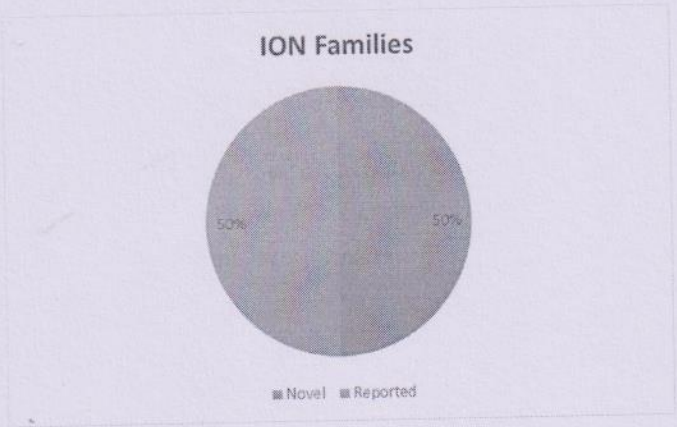


Fig 5: Variants identified in ION families (Whole genome analysis)



Manuscript under preparation:

- Complete mitochondrial genome sequencing in individuals with LHON negative for the common pathogenic mitochondrial DNA variants (manuscript under preparation)
- Rare mtDNA *ATP6* gene variations can cause isolated optic atrophy (manuscript under preparation)
- Biallelic variations in nuclear-encoded genes can cause and autosomal recessive LHON-like phenotype (manuscript under preparation)

iii) **Proposed utilization of the experience in India:**

Diagnostic and Research

The training which I gained from the Host Institute in analyzing and troubleshooting big genomics data set (Whole genome sequencing / Whole exome sequencing) in ION / LHON families will be useful in setting up a world class diagnostic service at my Parent Institute in India, to help diagnose patients with a suspected diagnosis of ION/LHON.

Translation:

Expertise gained by learning functional validation assays and CRISPR-Cas9 gene editing technology will be helpful to propose research projects with therapeutic intervention in LHON / DOA patients.

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